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(54) **VISCOELASTIC GELS AS NOVEL FILLERS**

VISCOELASTISCHE GELE ALS NEUE FÜLLMATERIALIEN

GELS VISCO-ÉLASTIQUES UTILISÉS EN TANT QU'AGENTS DE REMPLISSAGE NOVATEURS

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- **translation of priority document PCT/EP2010/005161 & comparison of application underlying opposed patent and priority document**

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Description

SUBJECT OF THE INVENTION

[0001] Viscoelastic gels as fillers prepared according to the processes as claimed.

FIELD OF INVENTION

[0002] Hyaluronic acid (HA) is a heteropolysaccharide consisting of alternating residues of D-glucuronic acid and N-acetyl-D-glucosamine.

[0003] HA is a straight-chain polymer with a molecular weight ranging between 50,000 and 13 x 10^6 Da, depending on the source from which it is obtained and the preparation methods used.

[0004] HA is present in nature in pericellular gels, in the ground substance of the connective tissue of vertebrates (of which it is one of the main components), in the vitreous humour and in the umbilical cord.

[0005] HA plays an important part in the biological organism as a structural and mechanical support for the tissues, and as an active component in the cell physiology of tissues such as skin, tendons, muscles and cartilage.

[0006] It is one of the main molecules in cartilage matrix, and also represents the main non-protein constituent of synovial fluid. As it is a strongly hydrophilic viscoelastic molecule, it gives the synovial fluid lubricant properties; HA has therefore been used in osteoarthritis for over 30 years, mainly to treat the associated pain.

[0007] HA also plays a crucial role in the tissue repair process from the structural standpoint (in the organisation of the extracellular matrix and regulation of its hydration), and as stimulating/regulating substance of a wide range of physiological processes wherein said polysaccharide acts directly and/or indirectly (clot formation, phagocyte activity, fibroblast proliferation, neovascularisation, re-epithelialisation, etc.) (Weigel P. et al., J Theoretical Biol, 1986:219-234; Abatangelo G. et al., J Surg Res, 1983, 35:410-416; Goa K. et al., Drugs, 1994, 47:536-566). As these properties have long been recognised, HA is also used to prepare dressings for the care of wounds, ulcers and skin lesions of various origins.

[0008] Hyaluronic acid is also used as a filler for wrinkles, furrows and small depressed areas of the face, and to increase the volume of the lips and cheeks, because it is immunologically inert, non-toxic, biodegradable and bioresorbable.

[0009] Treatment based on hyaluronic acid is indicated for the correction of:

- lip volume and contours
- furrows (e.g. nasolabial folds)
- remodelling of facial contours (e.g. cheeks and chin)
- wrinkles (e.g. glabellar lines and oral commissures)
- periorbital wrinkles
- fibrous post-acne scars
- fibrous post-traumatic scars
- soft tissue blemishes
- rhinoplasty scars.

[0010] Hyaluronic acid is not a permanent filler. This means that once injected, the product is gradually metabolised and resorbed by the body in times varying according to the area treated and the type of preparation used. The effect of filling and increased volume (or attenuation of wrinkles) is immediate, and only lasts a few weeks. The main products present on the market can be classified under the following categories, based on their different resorption times:

- rapid-resorption fillers (2-3 months),
- medium-term resorption fillers (5-6 months),
- slow-resorption fillers (1 year) such as Restylane Sub Q (QMed, EP0839159).

[0011] In the dermis, HA performs hydrating functions due to its high capacity to bind water, and structural functions as "scaffolding" because, by binding to other substances, it forms macromolecular complexes which render the skin compact.

[0012] The action mechanism therefore consists of immediate volumetric filling due to the viscoelastic properties of the product, and new collagen synthesis due to stimulation of the cutaneous fibroblasts.

[0013] However, HA is a natural polysaccharide which is rapidly broken down by the hyaluronidase enzymes present in connective tissue; in order to obtain fillers whose effect lasts for several months, HA is therefore subjected to crosslinking processes which improve its viscoelastic properties and increase its residence time. The fillers thus formed are crosslinked, for example, through BDDE (1,4-butanediol diglycidyl ether, Restylane®, BELOTERO® and Regenyal Idea) or DVS (divinyl sulphone, Hylaform®), which create bridges between the polymer molecules. However, increasing the degree of crosslinking progressively denatures the HA to the extent of profoundly modifying its chemical, physical and biological properties. Excessively crosslinked HA matrices present as particulate solids which are no longer recognised by the cells (and especially by the immune system) as HA; the polysaccharide is therefore perceived as a foreign body, which triggers inflammatory reactions with the formation of fibrotic capsules around it. Moreover, excessively crosslinked HA is unable to stimulate the dermal/cutaneous tissue regeneration induced, as known from well-established scientific results, by HA fragments (especially those with a low molecular weight) which have the effect of stimulating collagen synthesis by the cutaneous fibroblasts.

[0014] Fillers are also classified as resorbable or permanent. The resorbable type are the most biocompatible; they consist of hyaluronic acid or collagen, either modified or present in their native form, and are consequently resorbed within a year at most. The permanent type consists of synthetic polymers such as polyacrylamides, par-

ticular crosslinked molecules which form a stable gel when combined with water. The permanent type always remain *in situ* and are very useful for filling the lips, but their use is not recommended because acute inflammations are increasingly often caused by their cutaneous insertion, leading to the formation of fibrotic capsules around the filler, which is perceived as a foreign body and therefore toxic.

[0015] WO2008/068297 discloses a filler consisting of HA crosslinked with BDDE and HA as such in a ratio of 1:1 or 1:0,05, wherein the free HA is entrapped in the crosslinked network.

[0016] WO2009/073437 describes a gel formulation based on polysaccharides, including HA, preferably in the crosslinked form, associated with an inhibitor of the degradation of the polysaccharide itself. Among the various crosslinking systems, BDDE is also mentioned.

[0017] WO99/24070 discloses describes viscoelastic compositions in which HA is esterified and is possibly associated with HA autocrosslinked derivatives, and further associated with drugs or biologically active substances.

[0018] WO97/49412 describes compositions for the treatment of arthropathies consisting of autocrosslinked HA, optionally associated with HA as such.

[0019] It is disclosed a type of biomaterial as filler and/or as product for body shaping, formed by mixing two HA derivatives crosslinked in different but complementary ways, to obtain a skin/tissue substitute which allows immediate hydration (and consequently immediate filling) of the treated skin/tissue, while maintaining very long *in vivo* breakdown times to eliminate the need for repeated injections, thus reducing the side effects.

[0020] The biomaterials prepared according to the process to which the present invention relates present particular characteristics of biocompatibility identical to those of hyaluronic acid as such, but their biodegradability is different; when implanted *in vivo,* their residence time is much longer than that of unmodified HA, thus allowing immediate regeneration/reconstruction of dermal/cutaneous tissue which has lost its original compactness.

DETAILED DESCRIPTION OF THE INVENTION

[0021] The applicant has perfected two processes for preparing a type of biomaterial as filler and/or as product for body shaping based on mixing two HA derivatives with different but complementary characteristics to obtain a product for injection in the treatment of skin blemishes, in dermatology, in dermocosmetology and/or in aesthetic surgery, which produces:

1. immediate dermal/cutaneous hydration
2. immediate filling of the treated tissue
3. very long breakdown times *in vivo*
4. reduced side effects.

[0022] The biomaterials consist of:

- autocrosslinked hyaluronic acid (ACP), mixed with
- hyaluronic acid crosslinked with BDDE (HBC).

[0023] The ACP used in the present invention, prepared as described in EP 0341745, possesses a mean degree of crosslinking of between 4 and 5% and is preferably prepared using HA with a mean molecular weight (MW) of 200 KDa. When hydrated it presents as an autocrosslinked gel with no molecules foreign to the native polysaccharide, because it arises from the ester bond between the carboxyl and hydroxyl groups of the same polysaccharide chain and/or adjacent chains. It is therefore devoid of immunotoxicity, as biocompatible as native HA, highly moisturising, and easily degradable by hyaluronidases, releasing molecules with a low molecular weight able to stimulate collagen synthesis to improve the tone and elasticity of the cutaneous tissue.

[0024] ACP is the HA derivatives responsible for the immediate hydration (leading to instant dermal filling) elicited by the intradermal injection of the filler prepared according to the process which the present invention relates.

[0025] HA crosslinked with BDDE (a molecule containing epoxy groups for the formation of ethers on the primary hydroxyls of HA) contains the crosslinking molecule, and is therefore more resistant to enzymatic degradation as it possesses ether bonds which stabilise the polysaccharide, giving the product obtained a long residence time.

[0026] Mixing of the two species of crosslinked HA leads to the formation of a biomaterial which has biocompatibility characteristics identical to those of native hyaluronic acid, but a different biodegradability so that, when implanted *in vivo,* its residence time is much longer than that of unmodified HA, thus allowing the regeneration/reconstruction of dermal tissue which has lost its original compactness. The Applicant has also demonstrated that their association quite unexpectedly leads to an *in vivo* breakdown time much longer than that of the commercial reference fillers formed by the same type of HA crosslinked with BDDE, with a consequent increase in residence time. Finally, the Applicant discloses the use of the biomaterials prepared with the process according to the present invention as fillers and/or as products for body shaping in the treatment of skin blemishes, in dermatology, in dermocosmetology and/or in aesthetic surgery.

[0027] The chemically heterogeneous nature of the biomaterial allows the properties of the end product to be modulated by suitably varying the weight ratio between the constituents. The two HAs can be mixed in the ACP:HBC ratio of 10:90 to 90:10: the weight ratio will be selected on the basis of the desired final viscosity, which will depend on the site treated. If areas requiring implantation of large amounts of biomaterial are to be treated, as in the case of filling of the breasts, buttocks, cheeks

or chin, or deep expression wrinkles, the biomaterial used will preferably present good compactness, and therefore a viscosity suitable to obtain a gel with an excellent consistency and a low biodegradability rate; in this case the ACP:HBC mixture will be between 10:90 and 50:50, and preferably 25:75, because the product obtained by increasing the weight fraction of HBC is more suitable to perform a longer-lasting volume-enhancing effect. However, if lip furrows or fine forehead wrinkles are to be treated, the ACP:HBC ratio will preferably be between 90:10 and 50:50, as a higher fraction of ACP in the filler produces a material more suitable for biorevitalization of the skin and correction of fine lines, minor expression wrinkles and the like. Moreover, the needle must have a very high gauge; the gel must therefore be easily extrudable and less viscous than the one described above. The rheological properties of the product are consequently adjustable on the basis of the selected ACP:HBC ratio.

[0028] ACP/HBC composition being equal, the properties of the biomaterial can also be suitably modulated by means of a targeted selection of the vehicle in which it is prepared: for example, an ACP:HBC 50:50 weight mixture dispersed in saline solution (0.9% NaCl) will be more viscous than if it is dispersed in phosphate buffer at pH=6.95; consequently, for this specific mixture, saline solution is a more suitable medium for the formulation of products with a limited dispersion rate *in situ.* Materials consisting of a prevalence of HBC exhibit the opposite profile. The viscoelastic properties of the material consequently affect the performance of the product.

[0029] The present invention relates to the two biomaterials preparation processes described above: process **A** and process **B.**

[0030] The novel processes **A** and **B** are divided into two steps:

1. process for the production of the HBC derivative, and
2. process for mixing it with the ACP derivative.

[0031] The two steps lead to the production of products with a very high degree of purity. With the methods normally used for the production of HA crosslinked with BDDE, the purifications are performed by washing the mass of gel obtained, or by dialysis. In both cases, optimum purification efficiency may not be achieved due the nature of the gel matrix which, in view of its tendency to swell, incorporates large amounts of solvent. These gels have low mobility and transport capacity, and tend to precipitate as gelatinous gums. The precipitate thus obtained, isolated as a solid, has different solubility and rheology properties when rehydrated, especially swelling capacity, elasticity and homogeneity (essential characteristics for a filler), from the gel before purification.

[0032] However, the method hereinafter described by the Applicant as process **A** precipitates the product in the form of a finely divided powder, which is consequently easily washable. Moreover, the careful choice of reaction conditions produces, after isolation by precipitation and washing, a product with gel reconstruction capacity by means of rehydration and sterilisation which gives rise to a biomaterial having reproducible, well standardised characteristics of elasticity and homogeneity.

[0033] Process **B** does not include the step of precipitation of the HBC product as a powder; the purification and homogenisation of the gel (obtained after mixing HBC with ACP) is effected at the crushing step, which involves passing it through a filter with a particulate matter retention coefficient of between 25 and 150 μm. This step purifies the final gel and makes it perfectly homogenous.

[0034] The HA used in the present invention to prepare the derivatives described above (HBC, ACP) can derive from any source, such as extraction from cockscombs or fermentation, and have a mean molecular weight of between 400 and $3x10^6$ Da, preferably between $1x\ 10^5$ Da and $1x\ 10^6$ Da, and even more preferably between 200,000 and $1x\ 10^6$ Da.

[0035] Novel manufacturing process **A** comprises the following steps:

Synthesis of crosslinked HBC

[0036]

1. Dissolution in alkaline solution (preferably 0.15M - 0.35M NaOH) of diepoxide BDDE in a stoichiometric ratio of between 2.5 and 25% in moles, preferably between 5 and 15% in moles (depending on the intended use of the product; the higher the percentage of BDDE, the longer the residence time) of the repetitive units of hyaluronic acid, followed by
2. dispersion of HA in the solution referred to in the preceding paragraph, at room temperature. The HA concentration must be between 80 and 300 mg/ml, and the homogenisation time between 30 and 300 minutes.
3. Triggering of the reaction by heat activation, said solution being heated at a temperature of between 35 and 55°C for between 2 and 36 hours.
4. Extrusion of the mass obtained through a metal sieve, to reduce it to particles with a size of approx. 600 μm.
5. Hydration of gel by diluting it with water by a factor of 3 to 25, for a time of between 4 and 48 hours at a temperature of 4 to 24°C.
6. Correction of pH to neutral with an aqueous HCl solution having a concentration of 0.5 to 5 moles/l, preferably 1 to 2 moles/l.
7. Addition of 2.5 volumes of water-soluble organic solvent such as ethanol, methanol, isopropanol, n-propanol, dioxane, acetonitrile, acetone and/or mixtures thereof (preferably ethanol and acetone), until the product is obtained in the form of a precipitated powder.
8. Washing with organic solvents such as ethanol, methanol, isopropanol, n-propanol, dioxane, ace-

tonitrile, acetone and/or mixtures thereof (preferably ethanol and acetone), containing a water fraction of under 35%.

9. Drying under vacuum at a temperature of between 30 and 45°C for between 2 and 7 days, and in any event until elimination of the residual solvents under 400 ppm, to obtain a white HBC powder.

Mixing of ACP with HBC

[0037]

10. Mixing of the HBC powder with ACP powder in an ACP:HBC ratio of between 10:90 and 90:10 (depending on the use chosen, as previously described).

11. Hydration with saline solution or phosphate buffer, preferably saline solution (which may contain further excipients such as lidocaine), leading to a total HA concentration of between 12 and 27 mg/ml, preferably between 20 and 25 mg/ml, at a temperature of between 0 and 26°C.

12. Extrusion through a sieve with a mesh of between 50 and 500 μm, preferably between 100 and 250 μm. Said filtration is performed at room temperature, or at a temperature of between 25 and 65°C, preferably between 40 and 60°C.

13. Filling of syringes, preferably made of glass or polymer material, with the product obtained.

14. Heat sterilisation with saturated steam at a temperature of between 120 and 124°C (preferably 121.5 ±1°C) for at least 10 min.

[0038] Novel manufacturing process **B** comprises the following steps:

Synthesis of crosslinked HBC

[0039]

1. Dissolution in alkaline solution (preferably 0.15M - 0.35M NaOH) of diepoxide BDDE in a stoichiometric ratio of 2.5 to 25% in moles, preferably between 5 and 15% in moles (depending on the intended use of the product) of the repetitive units of hyaluronic acid, followed by.

2. dispersion of HA in the solution referred to in the preceding paragraph, at room temperature. The HA concentration must be between 80 and 300 mg/ml, and the homogenisation time between 30 and 300 minutes.

3. Triggering of the reaction by heat activation, said solution being heated at a temperature of between 35 and 55°C for between 2 and 36 hours.

4. Correction of pH to neutral with an aqueous HCl solution having a concentration of 0.05 to 1 moles/l, preferably 0.1 moles/l.

5. Hydration of gel by diluting it with water by a factor of 3 to 20 for a time of between 4 and 48 hours at a temperature of 4 to 24°C. This solution may contain further excipients, such as NaCl, phosphoric acid sodium or potassium salts, and lidocaine, preferably in the form of hydrochloride salt. Sodium salts (chloride or phosphate) have the function of maintaining the appropriate osmolarity of the product, and maintaining the pH at a value compatible with the tissues. In a preferred embodiment of the invention, NaCl is added in an amount such that the final solution contains a concentration of between 0.8 and 1.0% thereof, preferably 0.9%; the lidocaine hydrochloride, if present, is added in an amount such that the final formulation contains an amount of between 2.2 and 3.2 mg/ml thereof, preferably 2.7 mg/ml.

Mixing of ACP with HBC

[0040]

6. Mixing of the HBC gel with ACP powder in the ACP:HBC ratio of between 10:90 and 90:10 (in weight of the active ingredient) depending on the use chosen for the filler, as previously described. Alternatively, the ACP can be mixed with HBC starting with both components in gel form, using a suitable stirring system (preferably with an orbital blade) for a time of between 30 minutes and 24 hours at a temperature of between 0 and 26°C.

7. Crushing and homogenisation by passing through a filter with a particulate matter retention coefficient of between 25 and 150 μm, preferably between 40 and 110 μm. If the viscosity is excessive, the operation can be performed hot, at a temperature of between 25 and 65°C.

8. Filling of syringes, made of glass or polymer material, with the product obtained.

9. Sterilisation by heat from saturated steam at a temperature of between 120 and 124°C (preferably 121.5 ± 1°C) for at least 10 min.

[0041] Some examples of preparation of the filler with the processes according to the invention are described below, by way of example and not of limitation.

Example 1: Synthesis of HBC 500 (HA 500-730 kDa)

process A

[0042] 0.075 moles of HA with a molecular weight of 500-730 kDa, produced by fermentation, are dispersed in 215 ml of an 0.25M NaOH solution containing 1.41 ml of BDDE. The mixture is then heated to 42°C and reacted for 3 hours. The mixture is then hydrated for 24h with 300 ml of a solution containing a stoichiometric amount of HCl to adjust the pH to neutral. The total volume is made up to 750 ml and precipitated with 2.5 volumes of ethanol to obtain a filterable, decantable precipitate. The mixture

is washed with 75% ethanol until exhaustive purification, verified by measuring the specific conductivity of the washing solvents, which should be under 30 μS/cm, and dried under vacuum at 40°C for 5 days. The HBC 500 product is obtained with a weight yield of 87%.

Example 2: Synthesis of HBC 1000 (HA 1MDa)

process A

[0043] 1.60 g of HA with a mean molecular weight of 1 MDa, produced by fermentation, is dispersed in 20 ml of an 0.25M NaOH solution containing 75 μl of BDDE. The mixture is then heated to 42°C and reacted for 2 hours. The mixture is then hydrated for 24h with 20 ml of a solution containing a stoichiometric amount of HCl to adjust the pH to neutral. The total volume is made up to 75 ml and HBC is precipitated with 2.5 volumes of ethanol to obtain a filterable, decantable precipitate. The mixture is washed with 75% ethanol until exhaustive purification, verified by measuring the specific conductivity of the washing solvents, which should be under 30 μS/cm, and dried under vacuum at 40°C for 5 days. The product HBC 1000 is obtained with a weight yield of 90%.

Example 3: Synthesis of HBC 200 (HA 200 kDa)

process A

[0044] 2.55 g of HA with a mean molecular weight of 200 KDa, produced by fermentation, is dispersed in 20 ml of an 0.25M NaOH solution containing 63 μl of BDDE. The mixture is then heated to 42°C and reacted for 150 minutes. The mixture is then hydrated for 24h with 20 ml of a solution containing a stoichiometric amount of HCl. The total volume is made up to 75 ml and precipitated with 2.5 volumes of ethanol to obtain a filterable, decantable precipitate. The mixture is washed with 75% ethanol until exhaustive purification, verified by measuring the specific conductivity of the washing solvents, which should be under 30 μS/cm, and dried under vacuum at 40°C for 5 days. The product HBC 200 is obtained with a weight yield of 85%.

Example 4: preparation of ACP:HBC 500 gel, in the ratio of 50:50 process A

[0045] 1.00 g of HBC 500, prepared as described in example 1, is mixed with 1.00 g of HA ACP internal ester. The powder is hydrated with 100 ml of 0.9% weight/volume sterile saline solution at the temperature of 8°C for 16 hours. The gel obtained is heated to 48°C and filtered through a metal sieve with a mesh of 0.17 mm, and then distributed between 1 ml glass syringes, which subsequently undergo a sterilisation cycle with saturated steam at the temperature of 121°C for 10 minutes. A homogenous sterile gel suitable for local administration is obtained.

Example 5: preparation of ACP:HBC 1000 gel, in the ratio of 30:70 process A

[0046] 1.40 g of HBC 1000, prepared as described in example 2, is mixed with 0.60 g of HA ACP internal ester. The powder is hydrated with 100 ml of 0.9% w/v sterile saline solution at the temperature of 8°C for 16 hours. The gel obtained is heated to 48°C and filtered through a metal sieve with a mesh of 0.17 mm, and then distributed between 1 ml glass syringes, which subsequently undergo a sterilisation cycle with saturated steam at the temperature of 121°C for 10 minutes. A homogenous sterile gel suitable for local administration is obtained.

Example 6: preparation of ACP:HBC 500 gel, in the ratio of 25:75 process A

[0047] 1.875 g of HBC 500, prepared as described in example 1, is mixed with 0.625 g of HA internal ester ACP. The powder is hydrated with 100 ml of 0.9% w/v sterile saline solution at the temperature of 8°C for 16 hours. The gel obtained is heated to 48°C and filtered through a metal sieve with a mesh of 0.19 mm, and then distributed between 1 ml glass syringes, which subsequently undergo a sterilisation cycle with saturated steam at the temperature of 121°C for 12 minutes. A homogenous sterile gel suitable for local administration is obtained.

Example 7: preparation of ACP:HBC 1000 gel, in the ratio of 75:25 process A

[0048] 0.50 g of HBC 1000, prepared as described in example 2, is mixed with 1.50 g of HA internal ester ACP. The powder is hydrated with 100 ml of 0.9% w/v sterile saline solution at the temperature of 8°C for 24 hours. The gel obtained is heated to 42°C and filtered through a metal sieve with a mesh of 0.17 mm, and then distributed between 2 ml glass syringes, which subsequently undergo a sterilisation cycle with saturated steam at the temperature of 121°C for 12 minutes. A homogenous sterile gel suitable for local administration is obtained.

Example 8: preparation of HYADD:HBC 500 gel, in the ratio of 60:40 process A (comparative example)

[0049] 1.20 g of HBC 500 prepared as described in example 1 is mixed with 0.80 g of HA hexadecylamide (HYADD). The powder is hydrated with 100 ml of 0.9% w/v sterile saline solution at the temperature of 8°C for 24 hours. The gel obtained is heated to 52°C and filtered through a metal sieve with a mesh of 0.17 mm, and then distributed between 1 ml glass syringes, which subsequently undergo a sterilisation cycle with saturated steam at the temperature of 121°C for 11 minutes. A homogenous sterile gel suitable for local administration is obtained.

Example 9: preparation of HYADD:HBC 500 gel, in the ratio of 40:60 process A (comparative example)

[0050] 8.0 g of HA sodium salt with a mean molecular weight of 500-730 kDa, produced by fermentation, is dispersed in 40 ml of an 0.25M NaOH solution containing 0.44 ml of BDDE. The mixture is heated at 41.5°C for 2 hours 40 minutes. It is then hydrated overnight with 100 ml of an 0.1M HCl solution and 200 ml of water. 50 ml of a saturated solution of NaCl is added, and the mixture is left to swell overnight. The next day, 170 ml of acetone and 30 ml of saturated NaCl solution are added, and the mixture is precipitated by slowly adding one litre of ethanol. The precipitate is washed with the same solvent until the NaCl residues have been eliminated, then stove dried at 35°C under vacuum until the residual solvents have been eliminated. The HBC powder thus obtained is mixed in the ratio of 5:3 with HYADD, prepared as described in patent EP1853279. The mixed powders are hydrated with saline solution, leading to a total concentration of 20 mg/ml (corresponding to 12.5 mg/ml of HBC and 7.5 mg/ml of HYADD4). The product is left to swell overnight at 5°C, and the next day is filtered through a flat membrane with a nominal particulate matter retention rate of 100 μm. 1 ml glass syringes are filled with the product thus obtained and sterilised in a cycle with F0=13 at 121.5°C.

Example 10: Cutaneous filling and tolerability of HYADD:HBC gel in the intradermal rabbit administration model (comparative example)

[0051] The purpose of the experiment was to evaluate cutaneous filling, the onset of any macroscopic adverse events, and the tissue response elicited by HYADD:HBC gel (prepared as described in example 9) injected into the intradermal tissue of the rabbit, by comparison with the commercial filler BELOTERO®.

[0052] For said evaluation, the gels tested were administered intradermally to male NZW-KBL rabbits weighing 1.8-2.3 kg.

Experiment design:

[0053] The animals were anaesthetised by intravenous administration of ketamine and xylazine. 3 animals were used for each filler tested.

day 0: **T0**

[0054]

- Injection of samples (1 ml of hydrogel per sample) after shaving of the rabbits' backs;
- Measurement of the swelling on all rabbits and macroscopic observation for adverse events.

Day 7: **T7**

[0055]

- Measurement of swelling volume and macroscopic observation for adverse events.

[0056] The swelling volume was calculated with the formula:

$$(2/3 \times \pi) \times (r1) \times (r2) \times (r3)$$

where: (r1), (r2) and (r3) represent the width, length and height of the swelling respectively, measured with a caliper.

Results:

[0057] The filler did not cause any inflammatory event in the treated dermis.

[0058] The results obtained for the residence time are shown in Figure 1: the amount of swelling evaluated in the first week's treatment (expressed as mm^3) demonstrated that the gel according to the invention is capable of inducing a larger skin swelling volume than the control, which remains high even after 7 days, again to a much greater extent than the commercial filler used as comparator. This finding clearly confirms that the fillers immediately produce significant dermal hydration, and this effect is attributable to the presence of the HYADD derivative which, due to its chemical/rheological characteristics, has proved essential to promote immediate cutaneous filling which remains stable over time.

Example 11: Synthesis of HBC 500 (HA 500-730 kDa) process B

[0059] 18.75 g of HA sodium salt with a molecular weight of 500-730 kDa, produced by fermentation, is dispersed in 133 ml of an 0.25M solution of NaOH containing 885 μl of BDDE. The mixture is then heated at 45°C for 2.5 hours. The mixture is hydrated overnight with 0.62 l of a solution containing a stoichiometric amount of HCl, 2.65g of NaCl and 2.7g of lidocaine hydrochloride, under slow stirring.

Example 12: preparation of ACP:HBC 500 gel, in the ratio of 25:75 process B

[0060] 6.25 g of internal ester of hyaluronic acid ACP 200 is solubilised in 250 ml of a solution containing 4.4 g of NaCl under slow stirring. When hydration has been completed, the gel is combined with the gel obtained according to example 11 in a mixer equipped with a system for mixing semisolids, until homogenous. The gel obtained is extruded through a flat membrane filter with a nominal particulate matter retention rate of 70 μm. The

product thus obtained is introduced into glass syringes and sterilised in a cycle with F0=13 at 121.5°C.

Example 13: preparation of HYADD:HBC 500 gel, in the ratio of 25:75 process B (comparative example)

[0061] 6.25 g of HYADD hexadecylamide is solubilised in 250 ml of a solution containing 4.4 g of NaCl under slow stirring. When hydration has been completed, the gel is combined with the gel obtained according to example 11 in a mixer equipped with an orbital mixing system, until homogenous. The gel obtained is extruded through a flat membrane filter with a nominal particulate matter retention rate of 70 μm. The product thus obtained is introduced into glass syringes and sterilised in a cycle with F0=13 at 121.5°C.

Example 14: Synthesis of HBC 500 (HA 500-730 kDa) process B

[0062] 125 g of HA sodium salt with a molecular weight of 500-730 kDa, produced by fermentation, is dispersed in 1.33 1 of an 0.25M NaOH solution containing 9.4 ml of BDDE. The mixture is heated at 45°C for 2.5 hours. The mixture is hydrated overnight with 6.2 1 of a solution containing a stoichiometric amount of HCl, 26.5 g of NaCl and 27 g of lidocaine hydrochloride, under slow stirring.

Example 15: preparation of ACP:HBC 500 gel, in the ratio of 50:50 process B

[0063] 125 g of internal ester of hyaluronic acid ACP200 is solubilised in 2.5 l of a solution containing 44 g of NaCl under slow stirring. When hydration has been completed, the gel is combined with the gel obtained according to example 14 in a mixer equipped with an orbital mixing system with buffle and scraper. The gel obtained is extruded through a flat membrane filter with a nominal particulate matter retention rate of 45 μm. The product thus obtained is introduced into glass syringes and sterilised in a cycle with F0=13 at 121.5°C.

Example 16: Cutaneous filling and tolerability of ACP:HBC gel in the intradermal rabbit administration model

[0064] The experiment was performed as described in example 10, using gel prepared as described in examples 11-12, and comparing it with the Belotero® control and with a second commercial filler, Regenyal Idea.

[0065] For this experiment, the Applicant not only determined the skin swelling volume caused by the treatment but also evaluated the total residence time of the gel/filler prepared with the processes according to the invention by comparison with two well-known commercial fillers which represent the final comparator because both consist of HA crosslinked with BDDE.

[0066] The skin swelling in the treated rabbits was measured fortnightly (with macroscopic observation for adverse events) for a maximum of 96 days.

Results:

[0067] Figure 2 shows the results obtained: the findings described above were confirmed, namely immediate hydration of the treated dermis (mainly within the first 7 days) to a surprisingly greater extent than in the controls; moreover, the size of the skin swelling was more evident and the residence time longer than those of the two commercial comparators. At the end of the experiment, the filler prepared with the process according to the invention was still present, whereas the two controls had almost disappeared.

Claims

1. Process of mixing ACP with HBC prepared by a process comprising the following steps:

   a. dissolution in alkaline solution of diepoxide BDDE in a stoichiometric ratio from 2.5 to 25% in moles of the repetitive units of hyaluronic acid, followed by
   b. dispersion of hyaluronic acid (HA) in the solution referred to in paragraph a), at room temperature;
   c. triggering of the reaction by heat activation, the solution referred to in paragraph b) being heated at a temperature of between 35 and 55°C for between 2 and 36 hours;
   d. extrusion of the mass obtained through a metal sieve, to reduce it to particles with a size of approx. 600 μm;
   e. hydration of the gel obtained by diluting it with water by a factor of 3 to 20;
   f. correction of pH to neutral with an aqueous solution of HCl;
   g. precipitation with a water-soluble organic solvent until the product is obtained in powder form;
   h. washing with organic solvents containing water;
   i. drying under vacuum until the residual solvents under 400 ppm have been eliminated and an HBC powder is obtained;
   wherein the mixing process comprises the following steps:
   j. mixing of ACP powder with HBC powder in the ACP:HBC ratio of between 90:10 and 10:90;
   k. hydration with saline solution or phosphate buffer, leading to a total HA concentration of between 12 and 27 mg/ml;
   l. extrusion at a temperature of between 25 and 65°C through a sieve with a mesh of between 50 and 500 μm;
   m. syringe filling;

n. sterilisation by heat from saturated steam at a temperature of between 120 and 124°C for at least 10 min.

2. Process of mixing ACP with HBC prepared by a process comprising the following steps:

a. dissolution in alkaline solution of diepoxide BDDE in a stoichiometric ratio from 2.5 to 25% in moles of the repetitive units of hyaluronic acid, followed by
b. dispersion of HA in the solution referred to in paragraph a), at room temperature;
c. triggering of the reaction by heat activation, the solution referred to in paragraph b) being heated at a temperature of between 35 and 55°C for between 2 and 36 hours;
d. correction of pH to neutral with an aqueous solution of HCl;
e. hydration of the gel obtained by diluting it with water by a factor of 3 to 20;
wherein the mixing process comprises the following steps:
f. mixing of ACP gel or powder,_with HBC in gel form in an ACP:HBC ratio of between 90:10 and 10:90;
g. crushing and homogenisation by passing through a filter with a particulate matter retention coefficient of between 25 and 150 μm;
h. syringe filling;
i. heat sterilisation with saturated steam at a temperature of between 120 and 124°C for at least 10 min.

3. Process as claimed in claims 1 or 2, wherein the HA used for the preparation of the HBC and ACP derivatives has a mean molecular weight of between 400 and 3×10^6 Da, preferably between 1×10^5 Da and 1×10^6 Da, and more preferably between 200,000 and 1×10^6 Da.

4. Process as claimed in claim 2, wherein the ACP:HBC weight ratio is 25:75.

Patentansprüche

1. Verfahren zum Mischen von ACP mit HBC, das durch ein Verfahren hergestellt wurde, das die folgenden Schritte umfasst:

a. Auflösung von Diepoxid BDDE in alkalischer Lösung in einem stöchiometrischen Verhältnis von 2,5 bis 25 Mol% der Wiederholungseinheiten von Hyaluronsäure, gefolgt von
b. Dispersion von Hyaluronsäure (HA) in der Lösung, die in Absatz a) genannt ist, bei Raumtemperatur;
c. Auslösen der Reaktion durch Wärmeaktivierung, wobei die in Absatz b) genannte Lösung bei einer Temperatur von zwischen 35 und 55°C für zwischen 2 und 36 Stunden erwärmt wird;
d. Extrusion der erhaltenen Masse durch ein Metallsieb, um sie auf Partikel mit einer Größe von etwa 600 μm zu verringern;
e. Hydratisierung des erhaltenen Gels durch Verdünnen desselben mit Wasser mit einem Faktor von 3 bis 20;
f. Korrektur des pH auf neutral mit wässriger HCl-Lösung;
g. Präzipitation mit einem wasserlöslichen organischen Lösungsmittel, bis das Produkt in Pulverform erhalten wird;
h. Waschen mit organischen Lösungsmitteln, die Wasser enthalten;
i. Trocknung unter Vakuum, bis die restlichen Lösungsmittel unter 400 ppm entfernt wurden und ein HBC-Pulver erhalten wird;
wobei das Mischverfahren die folgenden Schritte umfasst:
j. Mischen von ACP-Pulver mit HBC-Pulver im Verhältnis ACP:HBC von zwischen 90:10 und 10:90;
k. Hydratisierung mit Kochsalzlösung oder Phosphatpuffer, was zu einer Gesamt-HA-Konzentration von zwischen 12 und 27 mg/ml führt;
l. Extrusion bei einer Temperatur von zwischen 25 und 65°C durch ein Sieb mit einer Maschenweite von zwischen 50 und 500 μm;
m. Spritzenfüllung;
n. Sterilisation durch Hitze aus gesättigtem Dampf bei einer Temperatur von zwischen 120 und 124°C für wenigstens 10 min.

2. Verfahren zum Mischen von ACP mit HBC, das durch ein Verfahren hergestellt wurde, das die folgenden Schritte umfasst:

a. Auflösung von Diepoxid BDDE in alkalischer Lösung in einem stöchiometrischen Verhältnis von 2,5 bis 25 Mol% der Wiederholungseinheiten von Hyaluronsäure, gefolgt von
b. Dispersion von Hyaluronsäure (HA) in der Lösung, die in Absatz a) genannt ist, bei Raumtemperatur;
c. Auslösen der Reaktion durch Wärmeaktivierung, wobei die in Absatz b) genannte Lösung bei einer Temperatur von zwischen 35 und 55°C für zwischen 2 und 36 Stunden erwärmt wird;
d. Korrektur des pH auf neutral mit wässriger HCl-Lösung;
e. Hydratisierung des erhaltenen Gels durch Verdünnen desselben mit Wasser mit einem Faktor von 3 bis 20;
wobei das Mischverfahren die folgenden Schritte umfasst:

f. Mischen von ACP-Gel oder -Pulver mit HBC in Gelform in einem Verhältnis ACP:HBC von zwischen 90:10 und 10:90;
g. Zerkleinerung und Homogenisierung durch Durchleiten durch ein Filter mit einem Retentionskoeffizienten für partikuläres Material von zwischen 25 und 150 μm;
h. Spritzenfüllung;
i. Hitzesterilisation mit gesättigtem Dampf bei einer Temperatur von zwischen 120 und 124°C für wenigstens 10 min.

3. Verfahren, wie in Anspruch 1 oder 2 beansprucht, wobei die für die Herstellung der HBC- und ACP-Derivate verwendete HA ein mittleres Molekulargewicht von zwischen 400 und 3×10^6 Da, vorzugsweise von zwischen 1×10^5 Da und 1×10^6 Da und bevorzugter von zwischen 200.000 und 1×10^6 Da, hat.

4. Verfahren, die wie in Anspruch 2 beansprucht erhalten werden, in denen das Gewichtsverhaltnis ACP:HBC 25:75 ist.

Revendications

1. Procédé de mélange d'ACP avec du HBC préparés par un procédé comprenant les étapes suivantes :

a. dissolution en solution alcaline du diépoxyde BDDE dans un rapport stœchiométrique de 2,5 à 25 % en moles des unités répétitives d'acide hyaluronique, suivie par
b. dispersion d'acide hyaluronique (HA) dans la solution citée dans le paragraphe a), à température ambiante ;
c. déclenchement de la réaction par activation à chaud, la solution citée dans le paragraphe b) étant chauffée à une température comprise entre 35 et 55 °C pendant 2 à 36 heures ;
d. extrusion de la masse obtenue à travers un tamis métallique, pour la réduire en particules ayant une taille d'environ 600 μm ;
e. hydratation du gel obtenu, par dilution de celui-ci avec de l'eau d'un facteur 3 à 20 ;
f. correction du pH pour l'amener à un pH neutre avec une solution aqueuse de HCl ;
g. précipitation avec un solvant organique hydrosoluble jusqu'à ce que le produit soit obtenu sous forme de poudre ;
h. lavage avec des solvants organiques contenant de l'eau ;
i. séchage sous vide jusqu'à ce que les solvants résiduels sous 400 ppm aient été éliminés et qu'une poudre de HBC soit obtenue ;
le procédé de mélange comprenant les étapes suivantes :
j. mélange de poudre d'ACP avec de la poudre de HBC dans un rapport ACP:HBC compris entre 90:10 et 10:90 ;
k. hydratation avec une solution saline ou un tampon phosphate, conduisant à une concentration totale de HA comprise entre 12 et 27 mg/ml ;
l. extrusion à une température comprise entre 25 et 65 °C à travers un tamis ayant une maille comprise entre 50 et 500 μm ;
m. remplissage d'une seringue ;
n. stérilisation par la chaleur à partir de vapeur d'eau saturée à une température comprise entre 120 et 124 °C pendant au moins 10 min.

2. Procédé de mélange d'ACP avec du HBC préparés par un procédé comprenant les étapes suivantes :

a. dissolution en solution alcaline du diépoxyde BDDE dans un rapport stœchiométrique de 2,5 à 25 % en moles des unités répétitives d'acide hyaluronique, suivie par
b. dispersion de HA dans la solution citée dans le paragraphe a), à température ambiante ;
c. déclenchement de la réaction par activation à chaud, la solution citée dans le paragraphe b) étant chauffée à une température comprise entre 35 et 55 °C pendant 2 à 36 heures ;
d. correction du pH pour l'amener à un pH neutre avec une solution aqueuse de HCl ;
e. hydratation du gel obtenu, par dilution de celui-ci avec de l'eau d'un facteur 3 à 20 ;
le procédé de mélange comprenant les étapes suivantes :
f. mélange de gel ou de poudre d'ACP avec du HBC sous forme de gel dans un rapport ACP:HBC compris entre 90:10 et 10:90 ;
g. broyage et homogénéisation par passage à travers un filtre ayant un coefficient de rétention de matière particulaire compris entre 25 et 150 μm ;
h. remplissage d'une seringue ;
i. stérilisation par la chaleur avec de la vapeur d'eau saturée à une température comprise entre 120 et 124 °C pendant au moins 10 min.

3. Procédé selon les revendications 1 ou 2, dans lequel le HA utilisé pour la préparation des dérivés HBC et ACP a une masse moléculaire moyenne comprise entre 400 et 3×10^6 Da, de préférence entre 1×10^5 Da et 1×10^6 Da, et plus préférentiellement entre 200 000 et 1×10^6 Da.

4. Procédé selon la revendication 2, dans lesquels le rapport pondéral ACP:HBC est de 25:75.

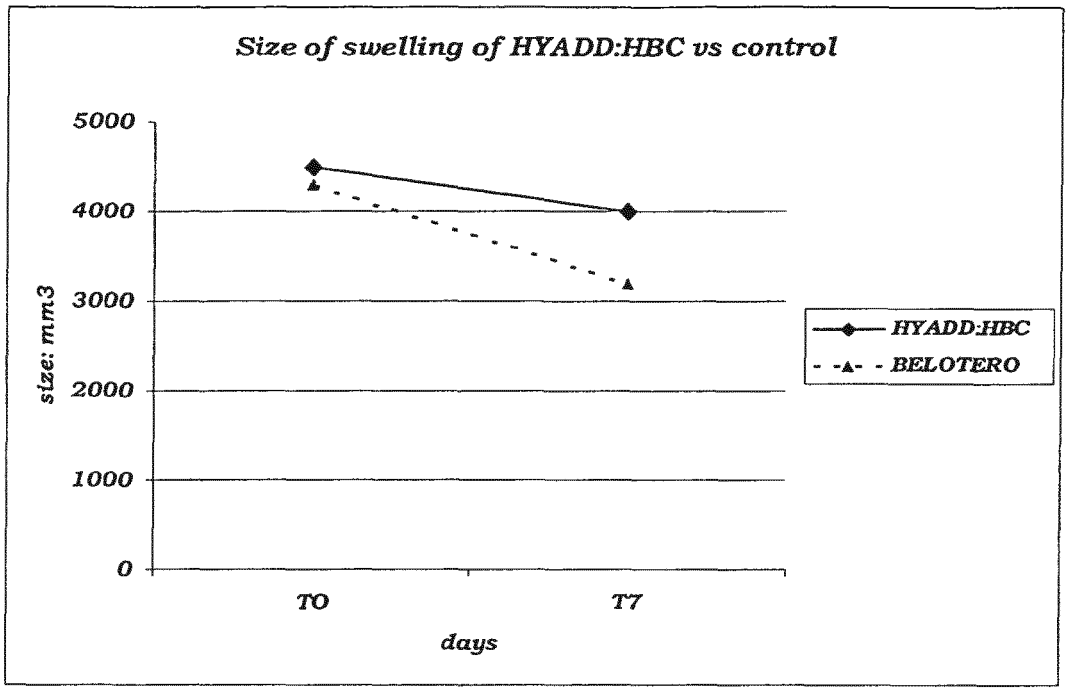

Figure 1 (comparative)

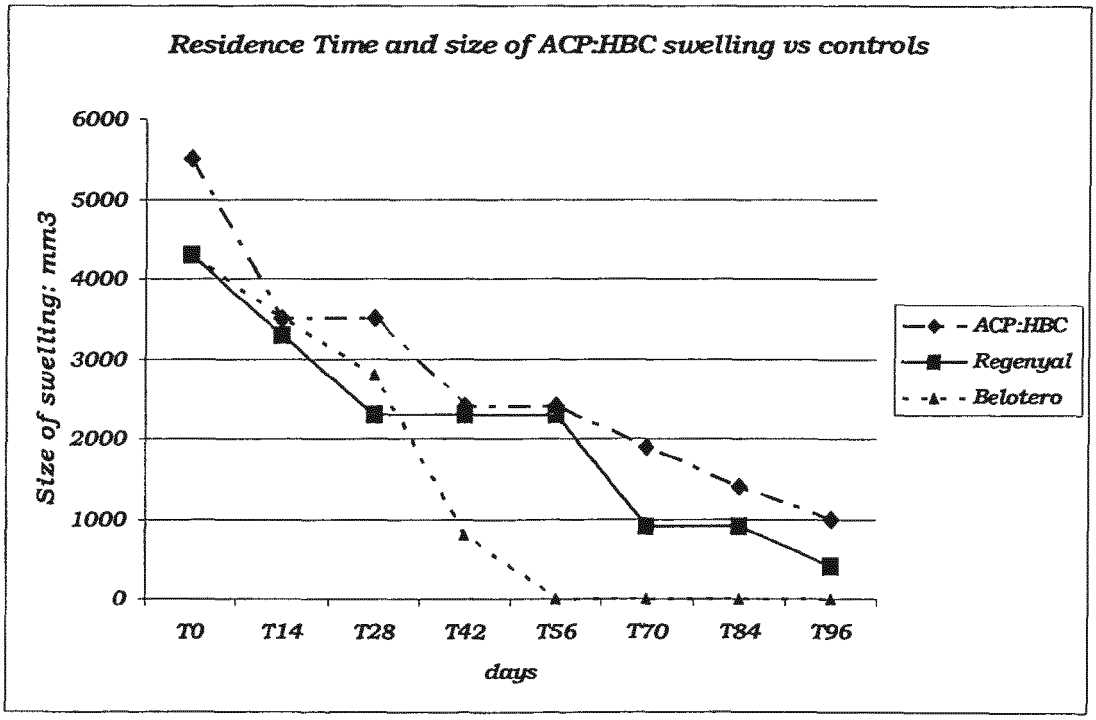

Figure 2

REFERENCES CITED IN THE DESCRIPTION

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